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GENE REGULATION OF METHANOGENESIS FROM ACETATE IN THE  
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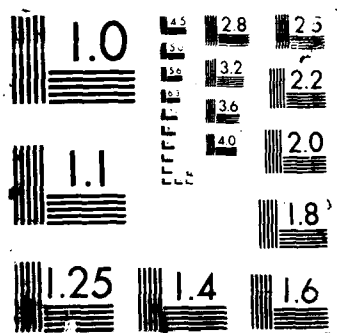
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The research objectives of the project were to develop genetic and molecular techniques that will permit the detection, isolation, and cloning of genes that are regulated during acetate catabolism in the Methanosarcina spp. These studies should provide a basis for examining the regulation of acetate utilization by the methanogenic Methanosarcina. We have concentrated on three areas in the two year period of the contract. They are: 1) refining plating techniques and selecting for auxotrophic mutants; 2) characterizing plasmid(s) from Methanosarcina spp.; 3) creating a gene library from Methanosarcina spp.; 4) creating oligonucleotide and antibody probes and probing the chromosomal library for CO dehydrogenase genes.

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Title: Gene Regulation of Methanogenesis from Acetate in the  
Acetotrophic Methane-Producing Archaeobacteria.

1. PROJECT GOALS

The goals of the research project were to characterize the regulation of genes in the Methanosarcina spp. that are involved in acetate catabolism by these acetotrophic archaeobacteria. Our approach was to develop basic techniques for studying molecular biology and genetics of Methanosarcina spp. by: i) developing plating techniques for mutant selection, ii) screening Methanosarcina spp. for plasmids which could be used in construction of gene shuttle vectors with the eventual goal of developing an gene transformation system, and iii) generation of genomic libraries of several methanogens followed by cloning of representative genes involved in acetate metabolism. Techniques developed in this study should aid in the study of other methanogenic archaeobacteria.

2. ACCOMPLISHMENTS

A. Cell Plating

In the first year of the project cell plating techniques were developed for the acetotroph Methanosarcina acetivorans. This species was one of the few Methanosarcina spp. that could grow as colony clones from single cells, a necessary requisite for genetic studies, in contrast to the multicellular aggregates typical of other methanosarcina species. Using the general approach devised by Bertani and Baresi (1) for the hydrogen oxidizing methanogen Methanococcus voltae, we developed a technique for plating M. acetivorans cells at 90-100% efficiency based on viable counts in liquid culture. We also demonstrated that colonies could be effectively replica plated.

During the second year we developed a technique for growing all species of Methanosarcina as single cells rather than cell packets or aggregates typical of these organisms. We refined the cell plating techniques to obtain high efficiency plating of these other species on solid medium. Since all the species of Methanosarcina could now be grown as single cells we were no longer restricted to the study of M. acetivorans. We began to concentrate our studies on M. thermophila because the biochemistry of acetate catabolism was the most extensively documented for this strain.

Methods were developed to mutagenize M. thermophila cells by UV irradiation. A reproducible kill curve could be obtained, based on log<sub>10</sub> survival of cells although a fraction of these cells, approximately 10<sup>-4</sup> to 10<sup>-5</sup>, appear UV resistant. A protocol has been standardized by which survivors of a 10<sup>-2</sup> cell killing UV dose are initially cultured on TMA plates and subsequently replica plated onto TMA, methanol, acetate plates, respectively to isolate colonies defective for acetate utilization. Replica plate procedures using velvet cloth or repeated transfer with wood applicator sticks yielded

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comparable results (figure 1). Mutant enrichment was tested for feasibility using either thiostrepton or novobiocin. Other antibiotics such as fusidic acid and puromycin await similar tolerance studies. Another approach mutant generation that is being evaluated is the exposure of wild type M. thermophila cells to substrate analogs.

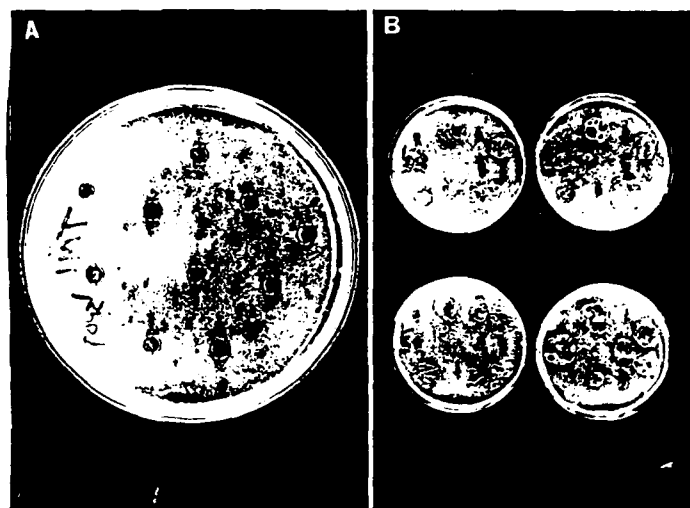


Figure 1. Plates of marine medium inoculated with the single cell form of M. thermophila by spotting inoculum on the surface (A) and by replica plating with a block (B). Bubbles in 7b are the result of partial lysis by older cultures; methane gas becomes trapped in the viscous lysate on the surface of the plate. Medium is described in figure 2 with the addition of 1.25% purified agar. Plates are poured and inoculated in an anaerobic glove box, then transferred to an anerobic jar under an  $N_2$ - $CO_2$  (4:1) atmosphere and incubated.

## B. Characterization of Plasmid DNA

In the first year we reported the screening of twelve strains of acetotrophic or methylotrophic methanogens for the presence of plasmid DNA. Plasmids were detected in three species of marine Methanosarcina and the plasmid from one of these species, M. acetivorans, was characterized in detail. A procedure was developed for the large scale purification of this plasmid. Resistance of the strains to various heavy metals is identical in plasmid bearing and plasmid free strains. Therefore, we as yet do not know the function of the plasmid.

Since the last annual report, we have completed the characterization of the plasmid pC2A and a manuscript has been accepted for publication in the Journal of Bacteriology. The plasmid is the

first to be isolated from an acetotrophic methanogen. It has properties that make it potentially useful for construction of a hybrid shuttle vector for genetic exchange purposes (figure 2). The relatively small size of the plasmid makes it easy to isolate and manipulate with minimum DNA shearing. It has several unique restriction sites including EcoRI and PstI that are commonly used for gene cloning. It also contains unique sites for the blunt end restriction endonuclease PvuII and SmaI which may be useful for insertion of other unique sites via DNA polylinker fragments. We are cloning pC2A into pBR322 with the goal of allowing us to amplify the plasmid in E. coli for a variety of studies.

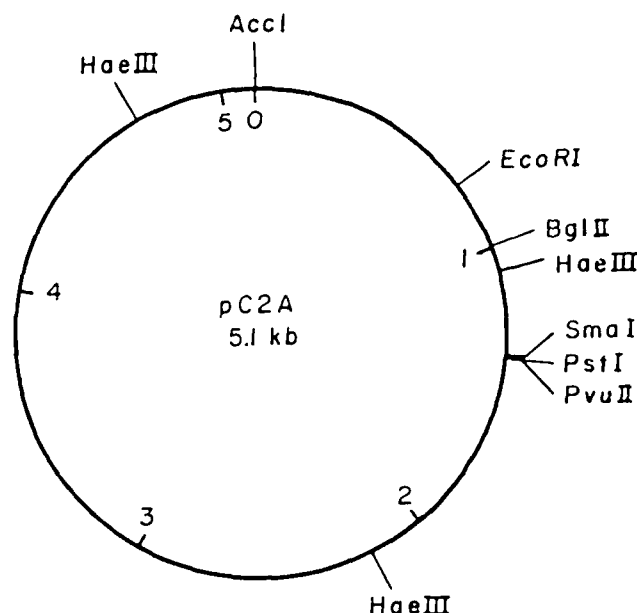


Figure 2. Map of plasmid pC2A from Methanosarcina aceticivorans.

### C. Cloning of CO Dehydrogenase Genes

#### 1) Isolation of Chromosomal DNA from M. thermophila

The preparation of large molecular weight DNA from Methanosarcina spp. has been a major technical barrier to performing gene cloning experiments in these species because the harsh mechanical methods needed to rupture the cells also shears the nucleic acids. In our last report we indicated that we had developed a technique to obtain intact DNA from Methanosarcina spp. We have since discovered that several Methanosarcina spp. in our culture collection can be adapted to grow in medium that contained marine salt concentrations. Concomitant with this adaption, strains cease to grow as multicellular aggregates and grow only as single cells (figure 3). These cells lack the typical heteropolysaccharide outer layer which makes them susceptible to gentle lysis with detergent (figure 3). This technique provides a method for isolating plasmid DNA and cellular RNA as well. As mentioned in our last report, we have isolated DNA from Methanosarcina

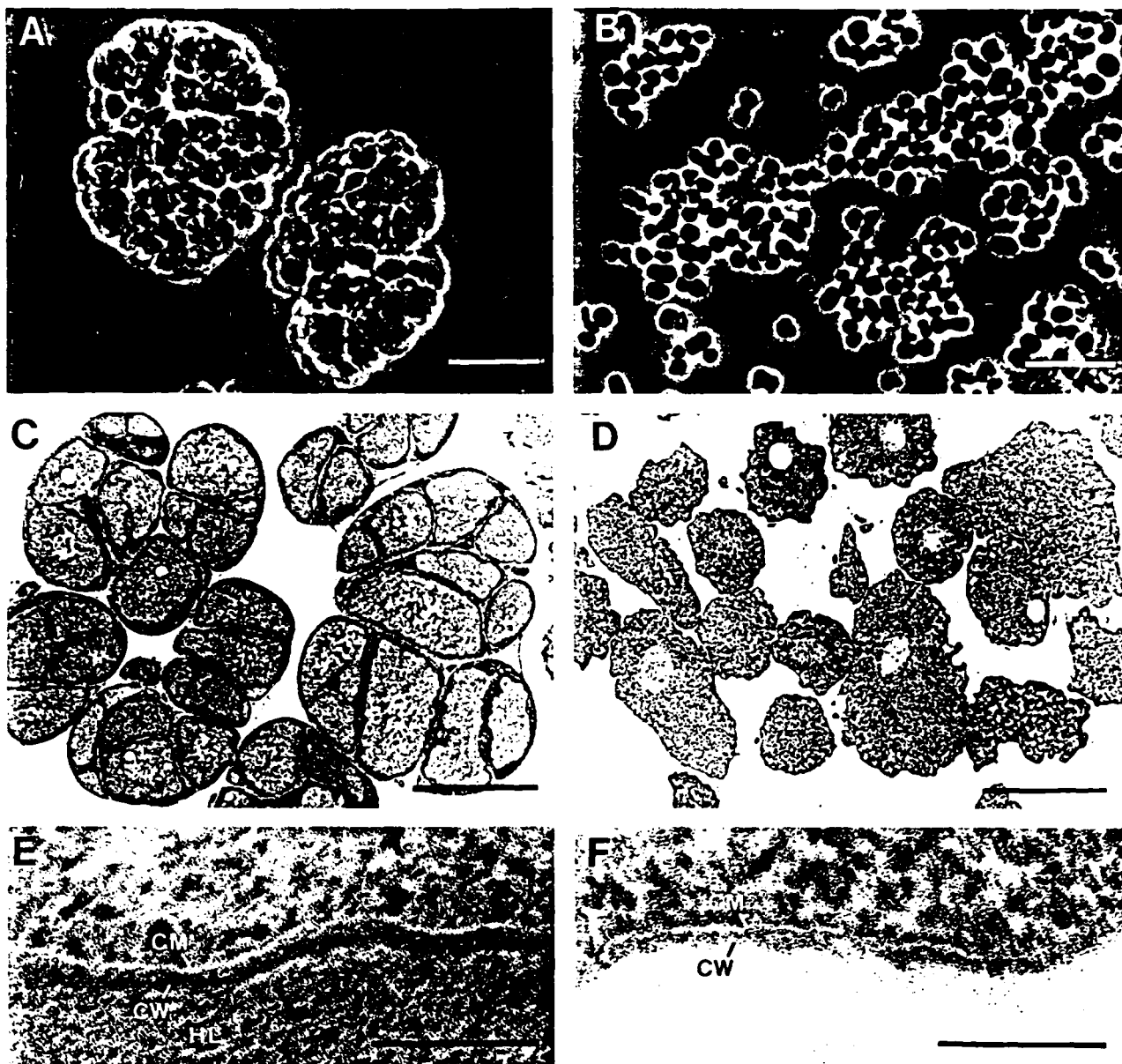


FIG. 3. Phase-contrast and thin-section electron micrographs of *M. thermophila*. Phase-contrast micrographs of *M. thermophila* show the large multicellular clumps that form in low-saline medium (A) and the single-cell form that is observed in marine medium (B); bars, 10  $\mu$ m. Thin-section electron micrographs of cells grown in low-saline medium show the close association of cells in multicellular clumps (C), while cells grown in marine medium lack the heteropolysaccharide outer layer (D); bars, 5  $\mu$ m. High-magnification thin-section electron micrographs of the cell-medium interfaces of low-saline-grown (E) and marine-grown (F) cells show the cytoplasmic membrane (CM), the cell wall layer (CW), and, in the case of low-saline-grown cells, the heteropolysaccharide layer (HL); bars, 0.1  $\mu$ m. Phase-contrast micrographs were made with an Olympus BH-2 microscope. Thin-section electron micrographs were prepared as previously described (18). Cells were fixed with 2% glutaraldehyde and 2% osmium tetroxide, dehydrated in a graded series of aqueous ethanol mixtures, and embedded in plastic resin for sectioning. Electron micrographs were made with a Hitachi H7000 electron microscope.

*thermophila*, *Methanosarcina mazei*, and *Methanosarcina barkeri* UBS and MS. The development of this technique has enabled us to pursue other aspects of the project because we are no longer restricted to working with the marine species *Methanosarcina acetivorans* as presented in the original proposal. We originally planned to use CO dehydrogenase from



M. thermophila provided by J.G. Ferry to produce probes for genomic libraries of M. acetivorans on the assumption that the genes for the protein would be sufficiently related in the two species. Now that we can isolate intact chromosomal DNA from M. thermophila (figure 4) we have continued our gene cloning studies on this species to ensure our chances of cloning CO dehydrogenase. A description on the application of the technique for M. thermophila has been published and a manuscript on the describing the single cell growth of the other species is in progress.

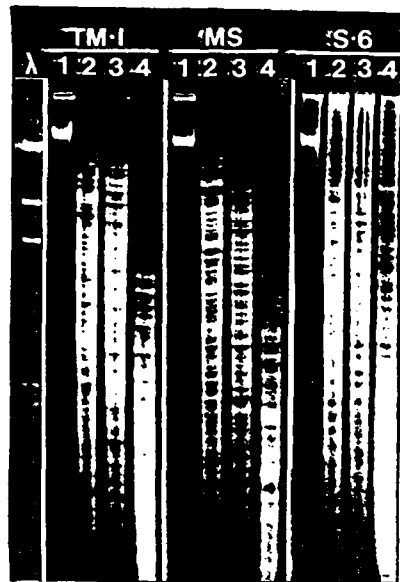


Figure 4. Agarose gel electrophoresis of whole and restriction enzyme-digested high molecular weight DNA from Methanosarcina thermophila TM-1, Methanosarcina barkeri MS, and Methanosarcina mazei S-6. Cultures were grown in marine medium, harvested by centrifugation and lysed with sodium dodecyl sulfate. Chromosomal DNA was purified by spooling precipitated DNA from ethanol. Lane  $\lambda$  is HindIII-digested lambda DNA included as a size standard. Lanes 1 through 4 are undigested (0.2 $\mu$ g), AccI, EcoRI, and HaeIII-digested (10 $\mu$ g) chromosomal DNA, respectively.

## 2) Construction of gene libraries from M. thermophila.

In our last report we described the generation of a gene library of M. acetivorans DNA. Since we can also culture M. thermophila as single cells for the isolation of chromosomal DNA, we have also generated genomic DNA libraries for this strain. An MboI partial digest of M. thermophila chromosomal DNA was fractionated (8 to 25 kilobases) before adding EcoRI linkers. The modified chromosomal fragments were then ligated into the phage vector  $\lambda$ gt11 to generate the library (titer of  $4.8 \times 10^9$  phage per ml, 98% insert frequency). This phage vector was selected because screening for genes can be effected by use of either oligonucleotide or antibody probes.

### 3) Amino acid sequencing of CO dehydrogenase and oligonucleotide synthesis.

As describe in our last report, we have determined the N-terminus amino acid sequences for two (19 and 89 kd) of the five subunits of purified CO dehydrogenase obtained from J.G. Ferry (V.P.I., Blacksburg, Virginia). Based on the primary amino acid sequence, we have designed and synthesized oligonucleotides for screening for the 19 kd subunit. The codon usage published for M. barkeri by Klein (2) was used to aid in the design of the probe. The phage library was screened by probing double plaque lifts on nitrocellulose filters. Filters from each plate were hybridized with  $\alpha^{32}\text{P}$ -end labelled oligonucleotides. Plaques that hybridized to both probes were considered positive and selected for further purification. We have obtained four positive clones and we are currently characterizing each clone to determine the size and properties of the chromosomal DNA insert.

Since the last annual report, we have gel purified the remaining three polypeptide subunits and are in the process of determining the N-terminal sequences of each. Oligonucleotide probes will be generated for each of these subunits and the remaining three genes of the CO dehydrogenase isolated. We have also examined the homology of the N-terminus sequences of CO dehydrogenase from M. thermophila with the sequences of CO dehydrogenase from other species to determine if regions of the proteins are conserved.

### 4) Antibody production and screening.

M. thermophila genomic libraries were screened with antisera directed against the 89 and 19 kd subunits of CO dehydrogenase. Protein subunits purified and used to raise antiserum in New Zealand rabbits. Titers of antiserum sufficient for probes were raise against both subunits. The  $\lambda$ gt11/M. thermophila library was plated on a lawn of E. coli y1090 and double plaque lifts were made with nitrocellulose filters. Filters were incubated with antisera and positive plaques were detected by the alkaline phosphatase color reaction. Six positive plaque clones were identified using the 89 kd antiserum. DNA purified from these clones contained inserts up to approximately 4 kb in length. We have subcloned these insert fragments into plasmid pUC18 for subsequent restriction mapping and other characterization studies. Since the gene for the 89 kd subunit has an estimated length of about 2500 bp, we will determine if we have the entire gene in the purified fragment by performing Western blots of in vitro transcription-translation reaction mixtures using E. coli extracts. The genomic library is currently being probed with antiserum to the 19 kd polypeptide. The remaining three subunits of CO dehydrogenase have also been gel purified and are being used to generate antisera specific to these subunits. The goal is to clone and characterize the genes for the remaining subunits of the enzyme.

### 3. CONCLUSIONS.

The following objectives have been achieved in the course of this project:

- 1) Techniques were developed for efficiently growing all Methanosarcina spp. on solid medium. A major limitation for conducting genetics on the Methanosarcina was eliminated by the discovery of cell growth conditions that cause cell aggregates to disassociate and grow as single cells, a condition necessary for performing genetic investigations with cell plating. Conditions for generating mutants for genetic regulatory studies were determined. A manuscript that describes portions of these studies is in preparation.
- 2) Acetotrophic methanogens were screened for the presence of plasmids. Three strains were found to contain plasmids. One of strains contained a plasmid designated pC2A, that was characterized and found to have features desirable for constructing a shuttle vector. A manuscript describing the properties of the plasmid has been accepted for publication.
- 3) Techniques were developed to obtain intact large molecular weight DNA from Methanosarcina spp. Gene libraries were constructed for two species and oligonucleotide and antisera probes for CO dehydrogenase were produced. Portions of one of the CO dehydrogenase genes has been cloned. The procedure for growing the Methanosarcina as single cells was devised. The results of these studies was recently published and one additional manuscript is in preparation that describes the properties of this alternative cell growth form.

### 4. PERSONNEL.

Dr. Kevin Sowers, a Postdoctoral Fellow, is supported in part by this grant. He was responsible for developing methods to culture the methanosarcina as single cells, the isolation and characterization of plasmid, and the isolation of intact chromosomal DNA from the various strains. He also was responsible for the gene library construction and gene cloning studies.

Dr. Surinder Sahota, a Postdoctoral Fellow, was also supported in part by this grant. He was responsible for the cell plating experiments in conjunction with Ms. Jane Boone.

Women or minorities - 1  
Non-citizens - 1 (citizen of England)

## 5. PUBLICATIONS AND REPORTS.

### Published Articles:

Sowers, K.R. and R.P. Gunsalus. 1988. Adaptation for growth at various saline concentrations by the archaeobacterium Methanosarcina thermophila. J. Bacteriol. 170:998-1002.

Sowers, K.R., and R.P. Gunsalus. 1988. Plasmid DNA from the acetotrophic methanogen Methanosarcina acetivorans. J. Bacteriol. (in press).

Manuscripts in Preparation: The following papers are in preparation and are the result of studies carried out during the last year of the Contract.

Gunsalus, R. P., S. Sahota, K.S. Sowers, and J. Boone. 1988. Single cell plating of the Methanosarcina spp. (in preparation)

Sowers, K.R., and R.P. Gunsalus. 1988. Interconversion between clumped and single cell growth in the Methanosarcina spp. (in preparation).

### Abstracts and Research Presentations:

Sowers, K.R. and R.P. Gunsalus. 1987. Ultrastructure of unicellular forms of Methanosarcina. Gordon Research Conference: Molecular Aspects of Methanogenesis, New Hampton.

Sowers, K.R. and R.P. Gunsalus. 1987. A unicellular phenotype exhibited by Methanosarcina thermophila. West Coast Bacterial Physiologists Conference, Asilomar.

Sowers, K.R. and R.P. Gunsalus. 1988. Adaptation for growth at various saline concentrations by Methanosarcina spp. Abstr. Ann. Mtg. Amer. Soc. Microbiol., 122, p. 18

Gunsalus, R.P., and K.R. Sowers. 1988. Genetics and Molecular Biology of the Acetate Utilizing Methanobacteria. Woods Hole Oceanographic Institution and the Marine Biological Laboratories. Woods Hole, MA. June 27, 1988.

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